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Human protein HC and its IgA complex are inhibitors of neutrophil chemotaxis

(enzyme-linked immunosorbent assay/α_1-microglobulin/monoclonal antibody/multiple myeloma/rheumatoid arthritis)

ENRIQUE MÉNDEZ*, JOSE LUÍS FERNÁNDEZ-LUNA*, ANDERS GRUBB†‡, AND FRANCISCO LEYVA-COBIÁN§

*Servicio de Endocrinología and §Servicio de Inmunología, Centro Ramón y Cajal, Carretera de Colmenar, Km. 9, 1, Madrid 34, Spain; and †Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-21401 Malmö, Sweden

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ABSTRACT **Protein HC, a heterogeneously charged low molecular weight glycoprotein, and its IgA complex were isolated from human plasma and urine. Plasma from individuals with monoclonal IgA populations was used as starting material for the isolation of the protein HC–IgA complex to obtain homogeneous complex populations. Neither low molecular weight protein HC nor its IgA complex in the concentrations 30 and 600 mg/liter influenced the random migration of normal human neutrophils. The chemotactic response of neutrophils to endotoxin-activated serum was, however, attenuated in a dose-dependent way by both low molecular weight protein HC and protein HC–IgA complex. Concentrations of protein HC and its IgA complex producing significant inhibition of the chemotactic response were found to occur in plasma from healthy and diseased individuals as well as in synovial fluid from patients with rheumatoid arthritis. These results suggest that protein HC and its IgA complex play physiological roles in the regulation of the inflammatory response.**

Protein HC, a heterogeneously charged complex-forming glycoprotein, is a low molecular weight protein recently isolated from normal human urine (1). Its charge heterogeneity is considerable, although its single polypeptide chain does not display any amino acid sequence variability (2). It carries an unidentified yellow-brown chromophore and is normally present in human plasma not only as the free low molecular weight protein but also to a great extent as a complex with IgA (1). The protein HC–IgA complex has been isolated and is composed of two light immunoglobulin chains, one normal heavy immunoglobulin α chain, and one polypeptide chain with a molecular mass of about 90,000 Da carrying both α-chain and protein HC epitopes (3). The protein HC epitopes occupy the carboxyl-terminal portion of the 90,000-Da chain (4).

Protein HC is closely related to two other recently described glycoproteins, α_1-microglobulin and α_1-microglycoprotein, isolated from the urine of patients with tubular dysfunctions (5–7). The amino acid sequences of protein HC and α_1-microglobulin have been published and are nearly identical except that the polypeptide chain of α_1-microglobulin is reported to be 15 residues shorter than that of protein HC (2, 8).

No physiological functions have been attributed to protein HC, α_1-microglobulin, α_1-microglycoprotein, or the protein HC–IgA complex. Protein HC and α_1-microglobulin have been described to be synthesized in hepatocytes (9, 10), whereas the production site for the protein HC–IgA complex is still unknown.

The present work was undertaken to investigate if protein HC and its IgA complex influence the chemotactic response of normal neutrophils to endotoxin-activated serum (EAS).

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MATERIALS AND METHODS

Isolation of Protein HC, Monoclonal IgA, and Protein HC–IgA Complexes. Blood plasma from a patient with multiple myeloma and concentrated (30 g/liter) monoclonal IgA and from a healthy blood donor with monoclonal IgA of low concentration (5 g/liter) was used as starting material for the isolation of two preparations of plasma protein HC and protein HC–IgA complex. The isolations were performed by immunosorption on columns containing anti-protein HC antibodies, followed by gel filtration as earlier described (3). Some isolations were carried out with immunosorbents containing polyclonal rabbit antibodies and some with immunosorbents containing monoclonal murine antibodies.

The monoclonal IgA components from the same starting material were isolated as described (3). They did not contain any protein HC–IgA complex as tested by crossed immunoelectrophoresis (11).

Urinary protein HC was prepared from the urine of a patient with tubular dysfunction as previously reported (1).

Protein concentrations were measured by a heated biuret–Folin assay (12) or by dissolving a known amount of lyophilized protein.

Monoclonal Antibodies Against Protein HC. The basic procedure of Köhler and Milstein (13) was used for the production of murine monoclonal antibodies against protein HC as described in detail elsewhere (14).

Quantitation of Protein HC and Protein HC–IgA Complex in Physiological Fluids. A competitive assay and a sandwich enzyme-linked immunosorbent assay using monoclonal antibodies against protein HC were used to measure the concentration of protein HC and protein HC–IgA complex in blood plasma and synovial fluid from healthy and diseased individuals (14). Quantitative crossed immunoelectrophoresis (15) was occasionally used to measure the concentration of protein HC and its IgA complex in some samples.

Preparation of Neutrophils. Blood from registered donors was collected in sterile tubes containing the anticoagulant sodium heparinate (14 international units/ml). Neutrophils were separated from erythrocytes and mononuclear cells by centrifugation in a Ficoll/Hypaque (Pharmacia and Nyegaard) gradient at $450 \times g$ for 40 min. Contaminating erythrocytes were lysed by hypotonic treatment, isotonicity was restored by addition of 0.3 M sodium chloride, and the neutrophils were washed twice in Hanks' balanced salt solution. The neutrophils were then diluted to 2×10^6 cells per ml with Gey's medium containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Chemotactic Assay. Neutrophil chemotaxis was evaluated by a modified Boyden chamber technique (16). EAS prepared as previously described (17) by using *Salmonella typhosa*

Abbreviations: EAS, endotoxin-activated serum; hpf, high-power field.

‡To whom reprint requests should be addressed.

0901 lipopolysaccharide W (Difco) and diluted with 7 parts of Gey's medium was invariably used as chemoattractant. The compartments were separated by polycarbonate membrane discs (Nuclepore, Bethesda, MD) with 3-μm pore size, and the chambers were incubated at 37°C in 100% relative humidity for 60 min.

The neutrophils (0.4×10^6 cells in 0.2 ml) were added to the upper compartment of the chemotaxis chamber with EAS containing various concentrations of protein HC or protein HC–IgA complex in the lower compartment. Random migration was tested by replacing EAS with Gey's medium and a possible chemoattractant activity of protein HC and protein HC–IgA complex by replacing EAS with Gey's medium containing various concentrations of protein HC or its IgA complex. For each protein concentration and control experiment the numbers of migrated cells within a square reticle in 10 high-power oil-immersion fields (hpf) were determined in each of three Boyden chamber membrane discs and the mean was calculated. The random migration value was always subtracted from the chemotaxis value. Inhibition of chemotaxis was expressed as follows: inhibition index (%) = 1 − (chemotaxis with protein HC or protein HC–IgA complex ÷ chemotaxis without protein HC or protein HC–IgA complex) × 100. The number of cells in the lower compartment was found to be insignificant in all experiments.

RESULTS

The influence of protein HC, isolated from plasma and urine, and its IgA complex on the random migration of neutrophils was tested by applying the proteins dissolved in Gey's medium in the lower compartment of the Boyden chamber in the concentrations 30 and 600 μg/ml. No influence on the random migration by these preparations could be observed. Neither did monoclonal IgA purified from the same plasma sample as protein HC and the protein HC–IgA complex influence the random migration of neutrophils.

When EAS was applied in the lower compartment of the chamber the neutrophils displayed a strong chemotaxis (with an average of 60 cells per hpf compared to 5 cells per hpf on random migration). When protein HC isolated from plasma or urine was added to the EAS a dose-dependent inhibition of the chemotaxis was observed (Fig. 1). Significant inhibition was obtained at a protein HC concentration of 25 μg/ml, and at 400 μg/ml the inhibition exceeded 80%. Virtually the same inhibition responses were obtained when the two protein HC preparations were heated to 56°C for 30 min before they were

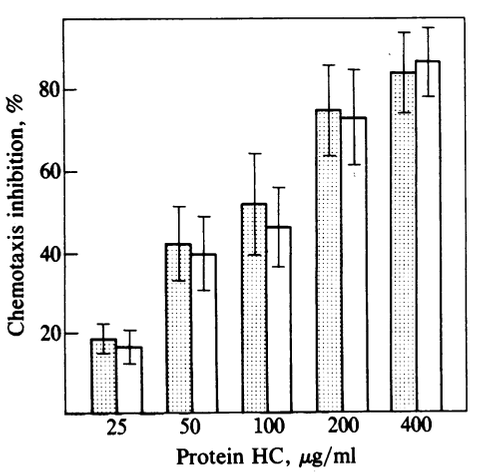

FIG. 1. Inhibition of neutrophil chemotaxis by protein HC. Open bars represent the mean inhibition (±SEM) by protein HC isolated from urine, and stippled bars represent the mean inhibition (±SEM) by protein HC isolated from blood plasma.

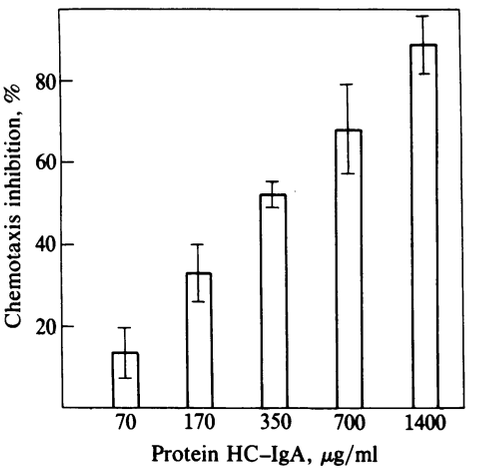

FIG. 2. Inhibition of neutrophil chemotaxis by protein HC–IgA complex. The bars represent the mean inhibition (±SEM) by protein HC–IgA complex isolated from blood plasma.

added to the EAS. Addition of bovine serum albumin to the EAS up to 1400 μg/ml did not cause any chemotaxis inhibition.

Isolated protein HC–IgA complex added to the EAS also caused a dose-dependent decrease of the chemotaxis (Fig. 2). Significant inhibition was seen at a final protein HC–IgA complex concentration of 70 μg/ml, and at 1400 μg/ml the inhibition exceeded 90%. The monoclonal IgA isolated from the same plasma sample as the protein HC–IgA complex also caused a significant inhibition of the chemotaxis, but it was only 50% on a weight basis of the inhibition caused by the protein HC–IgA complex.

Monoclonal anti-protein HC antibodies covalently linked to Sepharose beads were incubated with solutions of isolated protein HC and its IgA complex. When the supernatants of these mixtures were added to EAS the chemotaxis inhibition was found to be reduced by about 90% (Fig. 3).

Synovial fluid, which was found to inhibit neutrophil chemotaxis against EAS, was fractionated on a column containing covalently linked monoclonal antibodies against protein HC. When the column was washed with neutral buffer no substances inhibiting chemotaxis could be demon-

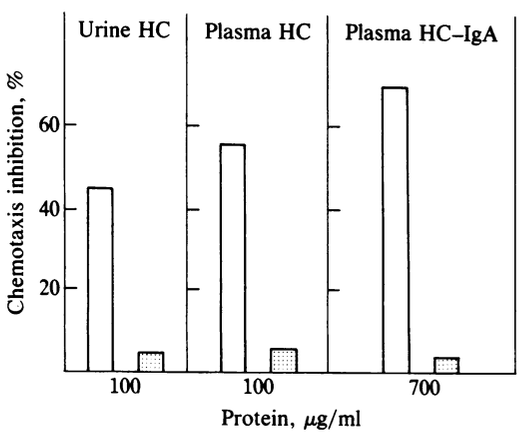

FIG. 3. Absorption of the neutrophil chemotaxis-inhibitory activity by monoclonal antibodies against protein HC. Solutions of protein HC isolated from urine and plasma and of protein HC–IgA complex isolated from plasma were incubated with monoclonal antibodies covalently linked to Sepharose beads. The chemotaxis inhibition was measured before (open bars) and after (stippled bars) incubation. More than 95% of the protein HC immunoreactivity was removed from the three solutions by the incubation as measured by enzyme-linked immunosorbent assays (14).

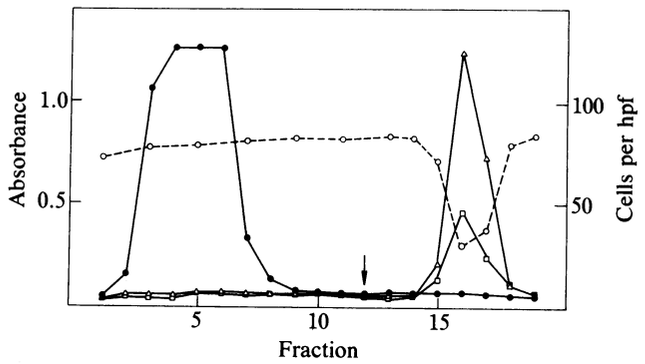

FIG. 4. Absorption of the neutrophil chemotaxis inhibitory activity in synovial fluid by monoclonal antibodies against protein HC. Three milliliters of hyaluronidase-treated synovial fluid was applied to a column containing monoclonal antibodies covalently linked to Sepharose beads. Fractions (2 ml) were collected and tested for their capacity to inhibit neutrophil chemotaxis. Neutrophil chemotaxis is expressed as number of migrated cells per hpf (○). The arrow denotes the replacement of the neutral starting Tris·HCl buffer, pH 7.4, with a glycine buffer, pH 2.2. Absorbance at 280 nm (●) was used to monitor the total protein concentration of the fractions, and two enzyme-linked immunosorbent assays (14) were used to measure the concentrations of protein HC (□) and protein HC–IgA complex (△) expressed as absorbance at 492 nm.

strated in the eluate. But on elution with pH 2.2 glycine buffer, chemotaxis-inhibiting substances were recovered from the column (Fig. 4). Sensitive enzyme-linked immunosorbent assays for quantitations of protein HC and protein HC–IgA complex detected these proteins only in the fractions capable of inhibiting chemotaxis (Fig. 4).

The concentrations of protein HC and protein HC–IgA complex in synovial fluid and blood plasma from several healthy and diseased individuals were measured by two enzyme-linked immunosorbent assays based upon monoclonal antibodies or by quantitative crossed immunoelectrophoresis. The results (Table 1) demonstrate that the concentrations of both protein HC and its IgA complex in most samples are within the ranges in which significant inhibition of neutrophil chemotaxis occurs.

DISCUSSION

The present investigation demonstrates that free low molecular weight protein HC isolated from blood plasma and urine inhibits neutrophil chemotaxis against EAS at physiologic concentrations. The protein HC concentration in blood plasma from healthy individuals is generally of a magnitude causing a significant but low inhibition of chemotaxis. But in some patients—e.g., those with impaired kidney function—the protein HC concentration may increase severalfold, and uremic patients might therefore suffer from a very substantial inhibition of neutrophil chemotaxis, contributing to the impaired immune defense of patients in this category (18).

The size and heat stability of protein HC distinguish it from most other described inhibitors of neutrophil chemotaxis, such as leukocyte inhibitory factor (19), chemotactic factor inactivator (20), polymeric and aggregated forms of IgA (21), and neutrophil migration inhibition factor from T lymphocytes (22, 23). Recent reports of Matzner *et al.* (24) and Matzner and Brzezinski (25) describe, however, a heat-stable chemotactic inhibitor in synovial and peritoneal fluid similar in size to protein HC. It is therefore of particular interest that the chemotaxis-inhibitory activity of synovial fluid, which was demonstrated by the *in vitro* assay of the present investigation, could be removed by passage of the synovial fluid through an immunosorbent column containing monoclonal antibodies against protein HC. Moreover, since the immunosorbed substances still retained the chemotaxis-inhibitory activity after elution and could be identified as protein HC and its IgA complex, it is quite possible that the chemotaxis-inhibitory activity described by Matzner *et al.* is carried by the protein HC molecule. As this activity recently was described to be missing in peritoneal fluid from patients with familial Mediterranean fever (26), it is also possible that such patients have abnormally low concentrations of protein HC or defective protein HC molecules without chemotaxis-inhibitory activity.

No investigation concerning a possible chemotaxis-inhibitory activity of α_1-microglobulin or α_1-microglycoprotein has been published, to our knowledge. But high concentrations of α_1-microglobulin have been shown to inhibit spontaneous leukocyte migration in agarose gels containing horse serum and the proliferative response of lymphocytes to tetanus toxoid (27).

The present study demonstrates that the chemotaxis-inhibitory effect of the low molecular weight protein HC also is carried by its IgA complex. In contrast to monomeric IgA, polymeric and aggregated IgA has been reported to suppress neutrophil chemotaxis (21). The inhibitory effect of the protein HC–IgA complex preparation cannot, however, be due to the presence of aggregated complexes, since the final isolation step of the monomeric complexes, carried out just before the chemotaxis assay, was a gel filtration in neutral buffer (3). Very little, if any, polymeric protein HC–IgA complex is present in human blood plasma (3, 28). The chemotaxis-inhibiting capacity of the monomeric monoclonal protein HC–IgA complex is probably carried by its protein HC part, since the molar inhibition by the complex, probably composed of one IgA molecule and one protein HC molecule, agrees with that of free protein HC. The inhibiting effect of the monoclonal IgA, isolated from the same plasma sample as the protein HC–IgA complex, was probably due to small amounts of aggregated IgA in this preparation, since monomeric native IgA does not inhibit neutrophil chemotaxis (21, 29).

Table 1. Concentrations of protein HC and protein HC–IgA complex in biological fluids

Sample category	Ref.	Assay	*n*	Protein HC, μg/ml		Protein HC–IgA complex, μg/ml	
				Mean	Range	Mean	Range
Plasma from	3	QCI	13	20	14–26	293	36–620
blood donors	14	ELISA	11	9	5–15	87	22–230
Plasma from patients with kidney failure	This work	QCI	10	160	84–220	154	29–244
Synovial fluid from patients with rheumatoid arthritis*	This work	ELISA	9	12	5–30	31	8–72

QCI, quantitative crossed immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay.
*After hyaluronidase treatment.

Protein HC and its IgA complex may inhibit neutrophil chemotaxis in two principal ways: either by a direct interaction with the neutrophil surface or by a modulation of the chemotaxins—e.g., complement factor C5a generated in EAS. The present investigation does not allow, however, any conclusions concerning the mechanism by which protein HC and its IgA complex inhibit neutrophil chemotaxis. Nor can it determine the relative importance of the polypeptide chain and the carbohydrate prosthetic groups of protein HC in the inhibitory process (30, 31).

In the initial phase of an inflammatory reaction a few neutrophils will concentrate at the inflammatory site due to the production of small amounts of chemoattractants by the agent provoking the inflammatory response. These first neutrophils will generate the formation of higher concentrations of chemoattractants—e.g., by discharging their specific granules containing a potent proteinase capable of releasing the chemotactic fragment C5a from complement factor C5 (32). More neutrophils will therefore accumulate at the inflammatory site and induce still higher concentrations of chemotaxins. Thus the inflammatory response would have a noxious self-amplifying character unless regulatory mechanisms were operating.

The present investigation suggests that protein HC and its IgA complex may be parts of such a regulatory mechanism by serving as physiological inhibitors of neutrophil chemotaxis. In healthy individuals the plasma concentration of protein HC and its IgA complex appear to give rise to a small but significant inhibition, whereas the high concentration levels in some patients with, e.g., kidney dysfunctions may severely impair the inflammatory response. Other patients—e.g., those with familial Mediterranean fever—may have too little or inactive protein HC and therefore suffer from too frequent and excessive inflammatory responses.

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